

Immunology Series No.10
Procedural Guide

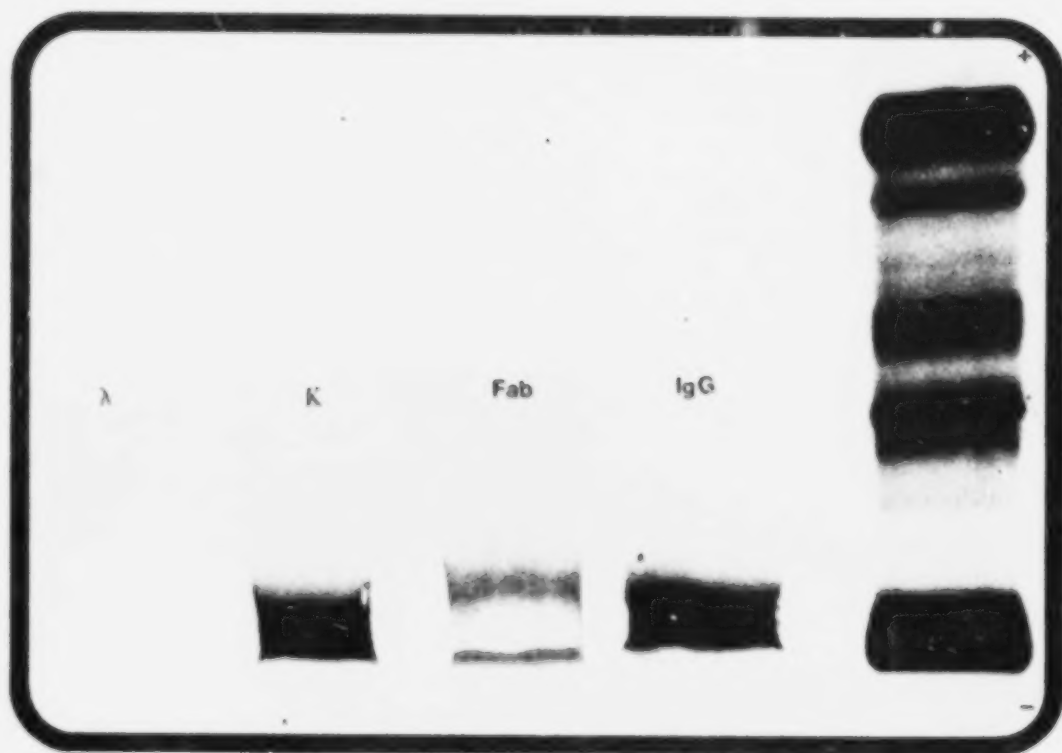
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Immunofixation Electrophoresis



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IMMUNOFIXATION ELECTROPHORESIS

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December 1981

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Centers for Disease Control

**Laboratory Improvement Program Office
Laboratory Training and Consultation Division
Atlanta, Georgia 30333**

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ACKNOWLEDGEMENTS

The authors express their appreciation and thanks to Dr. Robert F. Richie, Medical Director, Foundation for Blood Research, Scarborough, Maine, for his critical review of the manuscript and for providing time for one of us (JJC) to visit his laboratory. Special thanks to Mrs. Judy Howard, Foundation for Blood Research, for her time and instruction in the use of immunofixation.

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INTRODUCTION

Immunofixation electrophoresis refers to the fixation, in gel or paper, of one of the reactants (generally the antigen) in an antigen-antibody system by the other reactant (antibody).

Techniques described by Afonso (1), as quantitative immunoelectrophoresis, and by Wilson (20), as direct immunoelectrophoresis, can be considered as the progenitors of immunofixation electrophoresis. These procedures involved the performance of standard agar electrophoresis after which polyvalent antiserum was spread over the gel surface, rather than being placed in troughs as is done in routine immunoelectrophoresis. This technique was shown to enhance precipitation patterns but did so at the expense of resolution, making the results difficult to interpret. Alper and Johnson (2), described a modification of Afonso's basic technique; this consisted of antigen fixation by precipitation with monospecific antiserum after high-resolution agarose gel electrophoresis. Alper and Johnson termed their technique immunofixation electrophoresis. Immunofixation electrophoresis (IFE), or simply immunofixation, is presently the most commonly used term applied to this technique. Other terms which have been used to describe this procedure include: quantitative immunoelectrophoresis, electroimmunofixation, immunodisc electrophoresis, and immunocore electrophoresis.

Since its initial description, immunofixation has been increasingly accepted as a sensitive, relatively easy, and versatile method for detecting specific protein fractions. This technique combines the specificity and sensitivity of immunoprecipitation and the high resolution of electrophoresis to give a method of practical usefulness not only for the research laboratory, but also for the clinical laboratory. Immunofixation has been used successfully for the study of polymorphism (8), for genetic typing (7,16), for identification of serum protein fractions (3,4,11), and, more recently, for the study of human monoclonal proteins (5,12,13,17,18,19).

The basic principle of immunofixation electrophoresis resides in the fact that the immune complexes of antigen and

precipitating antibody become trapped in the gel's pores so that all protein except the complexes can be washed away. In practice, samples of serum or other fluids, appropriately diluted if need be, are subjected to electrophoretic separation in agarose, cellulose acetate, or acrylamide gels (6, 9, 10). Immediately after the separation procedure, high-titered monospecific antisera are used to precipitate the separated protein fractions. These antisera are applied to the gel in such a manner as to cover the area where the proteins of interest are known to have migrated. To accomplish this, an appropriately sized strip of cellulose acetate is saturated with monospecific antiserum, the excess antiserum is drained off, and the strip is applied to the gel surface. Strips saturated with different antisera may be used on the same gel; however, such strips must be positioned so as not to come into contact with one another.

During the incubation period, the monospecific antiserum applied to the gel surface diffuses into the gel to react with antigen at the site to which it has migrated and forms immune complexes. Provided that the monospecific antiserum is present in excess or near equivalency, the antigen-antibody complexes remain insoluble and too large to be washed out of the gel's pore structure. Thus, following the removal of the cellulose acetate strips, any unprecipitated proteins remaining in the gel can be washed out, leaving only the immunoprecipitates. These are then visualized by staining with appropriate protein stains.

The step-by-step procedure which follows is adapted from that by Ritchie and Smith (15,17) in which immunofixation electrophoresis is performed on agarose gel layered on a Mylar support. The procedure is directed specifically at the application of immunofixation electrophoresis to the study and identification of human monoclonal proteins in immunoglobulinopathies.

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I. MATERIALS REQUIRED

A. Reagents

1. Barbitol-HCl buffer, pH 8.6
2. Agarose, electroendosmosis (-mr) 0.13-0.19
3. Bromphenol blue stain
4. Coumassie brilliant blue stain, R-250
5. Alcohol-acetic acid destain solution
6. Sera:
 - a. Normal human serum
 - b. Patients' sera

B. Equipment

1. 101- x 203- x 5.0-mm glass plates
2. 205- x 110- x 1.0-mm plastic U-frame (Bio-Rad Laboratories, Richmond, California)*
3. Flexible polyester film, 203-mm width, 0.1-mm thick; one side is hydrophilic and the other is hydrophobic (GelBond™, FMC Corporation, Marine Colloids Division, Rockland, Maine)*
4. Paper clamps for holding glass plates, polyester film, and U-frame together
5. Sample application foil (LKB Instruments, Inc., Rockville, Maryland)
6. Cellulose polyacetate strips, 2.5- x 15.2-cm (Gelman Sepraphore III Cellulose, Gelman Sciences, Inc., Ann Arbor, Michigan)
7. Wicks, 200- x 250-mm (Ultra Wicks, Bio-Rad Laboratories)
8. Capillary tubes
9. Erlenmeyer flasks, screw cap, 500-ml, 1000-ml, 2000-ml
10. Serologic pipettes, 5-ml, 10-ml, 25-ml
11. Graduated cylinders, 100-ml, 500-ml
12. Volumetric flasks, glass stopper, 500-ml, 1000-ml, 2000-ml
13. Covered staining trays
14. Magnetic stirrer and bars
15. Tripod with asbestos pad

16. Bunsen burner

NOTE: Alternately, an electric heater with magnetic stirring may be used in place of items 14-16.

17. Interval timer

18. Hot air drying oven

19. Marking pen

20. Test-tube racks

21. Electrophoresis apparatus

- a. Electrophoresis chamber, with a glass cooling plate.

- b. Thermostatic circulator with circulating flow rate of 1-2 liters/min. and adjustable between 0°-20°C.

NOTE: Alternately, cooling of the glass plate with circulating water from a cold water tap can be used.

- c. Power supply, with a capability of 300 V and capacity of 150 mA per electrophoresis cell.

22. Refrigerator, 4°C.

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

II. PREPARATION OF REAGENTS

A. Barbital-HCl Buffer, pH 8.6

Chemicals needed:

Sodium barbital, ($\text{NaC}_8\text{H}_{11}\text{N}_2\text{O}_3$)

Calcium lactate, $\text{Ca}(\text{C}_3\text{H}_5\text{O}_3)_2 \cdot 5\text{H}_2\text{O}$

1N HCl

Distilled water

1. Prepare stock solutions as follows:

Solution A:

- Add approximately 1000 ml of distilled water to a 2-liter volumetric flask and heat over a Bunsen burner or electric heater until water is near boiling.
- Add 206.18 gm of sodium barbital to the flask and mix thoroughly to dissolve.
- Add distilled water (q.s.) to 2 liters and allow solution to cool to room temperature. This solution may be stored in a tightly closed bottle at room temperature ($20^\circ\text{--}26^\circ\text{C}$) for several weeks; however, storage at 4°C is preferable. Discard at the first sign of contamination.

Solution B:

- Add 86.2 ml of 11.6N HCl to a 1-liter volumetric flask.
- Add distilled water (q.s.) to 1 liter, stopper, and mix thoroughly. After mixing, a solution of 1N HCl is obtained.

NOTE: A HCl solution, at a concentration of 1N, may be obtained from a commercial source.

2. Prepare half-strength barbital-HCl buffer, pH 8.6, as follows:

- Add 0.62 gm of calcium lactate to a 2-liter volumetric flask.
- Add 174.2 ml of solution A and 12.9 ml of solution B to the flask. Mix thoroughly to dissolve the calcium lactate.
- Add distilled water (q.s.) to 2 liters, stopper, and mix.
- Check the pH with a prestandardized pH meter. The pH should be 8.6 ± 0.05 . If the pH is below 8.55, adjust with 1N NaOH. If the pH is above 8.65, adjust with 1N HCl.

- e. Store this final *working buffer* in a tightly closed bottle at 4°C. It may be stored for several weeks, but check pH before using. Discard at the first sign of contamination or change in pH.

B. 1.2% Agarose in Barbital-HCl Buffer

Chemicals needed:

Agarose, electroendosmosis (~mr) 0.13-0.19

Barbital-HCl buffer, pH 8.6

1. Add 98.8 ml of *half-strength barbital-HCl buffer* to a 250-ml Erlenmeyer flask.
2. Add 1.2 gm of agarose to the buffer.
3. Place the flask on an asbestos pad over a Bunsen burner, or on an electric heater with magnetic stirring, and heat the solution until agarose is completely dissolved, as indicated by complete clarity of the solution.

NOTE: Too much heating will turn the solution yellow. Keep the flask loosely covered during heating to retard evaporation. Once agarose is in solution, remove flask from heat and let cool to approximately 60°C before pouring plates. If the solution is too hot, it will cause the polyester film to buckle and warp.

C. Coumassie Brilliant Blue Stain

Mix:

Coumassie brilliant blue, R-250	4.0 gm
Ethanol (absolute)	800.0 ml
Glacial acetic acid	200.0 ml
Distilled water	1000.0 ml

Filter the solution and store at room temperature in a tightly closed bottle.

D. 0.4% Bromphenol Blue Stain Solution

Mix:

Bromphenol blue	0.4 gm
Barbital-HCl buffer, pH 8.6, half-strength	100.0 ml

E. Destaining Solution

Mix:

Ethanol	400.0 ml
Glacial acetic acid	100.0 ml
Distilled water	500.0 ml

PART A
Agarose Gel Electrophoresis

2-A

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III. PREPARATION OF AGAROSE PLATES

- A. Determine the number of plates needed to perform the electrophoresis screening test as follows:
 1. Count the number of patients' sera to be screened.
 2. Thirteen patients' sera, or twelve patients' sera and a normal human serum control, can be screened on one plate. Therefore, divide the *total* number of sera to be screened by thirteen to obtain the number of plates needed.
- B. Estimate the volume of 1.2% agarose solution needed for the plates determined above, as follows:
 1. Each plate is layered with 15 ml of 1.2% agarose. Therefore, multiply the number of plates to be made by 15 to determine the volume of agarose needed.
 2. Increase the volume obtained in the step above by a few milliliters to facilitate pipetting.
- C. Prepare the desired volume of 1.2% agarose in barbital-HCl buffer, as described on page 6.
- D. For each plate to be poured, prepare a gel mold as follows (see figure 1):
 1. Cut a piece of GelBond™ polyester film, 115- x 203-mm for each plate.
 2. Set aside 2, 101- x 203- x 5.0-mm glass plates for each agarose plate to be prepared.
 3. Wet one side of a glass plate with a small volume of distilled water and place a precut sheet of GelBond™ polyester film on the wet plate with its *hydrophilic side up*.

NOTE: This is tested by noting the behavior of a drop of water on both sides of the film; the water will "bead" on the hydrophobic side, whereas it will "spread" on the hydrophilic side.

No air bubbles should remain between the glass plate and the polyester sheet.

4. Place a plastic U-frame on top of the sheet and then the second glass plate, and clamp them together with strong paper clamps along the bottom and the edges so that you

have, from bottom to top, a mold consisting of a glass plate, polyester film, a plastic U-frame, and a second glass plate.

- E. Position the gel mold vertically on a flat surface with the open end of the mold up (figure 2).
- F. Light a Bunsen burner and place it close to the working area.
- G. Prewarm a 25-ml pipette by passing it through the flame of the burner, and draw up 15 ml of the hot 1.2% agarose which has been allowed to cool to 60°C.
- H. Quickly pipette the agarose into the mold. Keep the pipette at a 45° angle to the level of the mold and allow the agarose to flow out in one step (5-10 seconds) as a continuous stream between the GelBond™ film and the top glass plate. Any air bubbles should move rapidly upwards as the mold is filled (figure 3).
- I. Allow the agarose to solidify, approximately 20 minutes, at room temperature.
- J. Trim excess GelBond™ film from upper end of the gel mold and seal the open upper end of the mold with waterproof tape. The two glass plates and plastic U-frame must remain intact until the agarose plate (GelBond™ sheet layered with 1.2% agarose, 1.0-mm-thick) is ready for use.
- K. Place intact gel molds in a water saturated atmosphere (humidity chamber) and store at 4°C for at least 16 hours before use. Agarose plates can be stored for at least 1 week in a humidity chamber at 4°C.

NOTE: An alternate storage procedure: Remove the clamps from the gel molds, individually wrap them tightly with a wet paper towel and plastic wrap, and store them at 4°C.

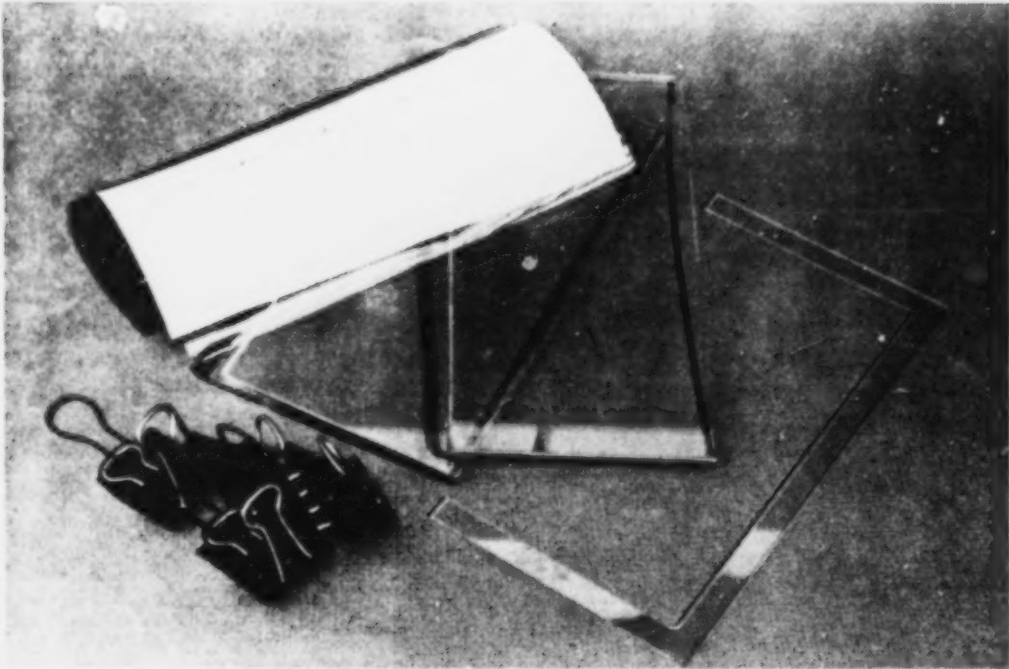


FIGURE 1. A view of the mold for preparing agarose gel plates before assembly. From left to right, the details are: clamps; sheet of GelBond™ polyester film; 2, 5.0-mm-thick glass plates (101- x 203-mm); and a 1.0-mm-thick plastic U-frame (110- x 205-mm).

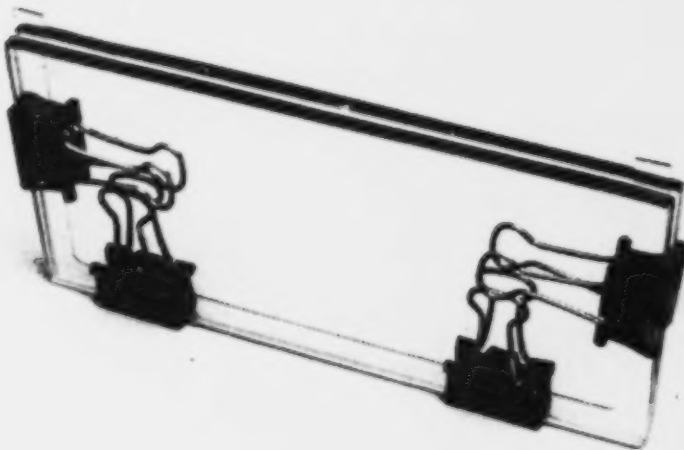


FIGURE 2. Assembled agarose gel mold.

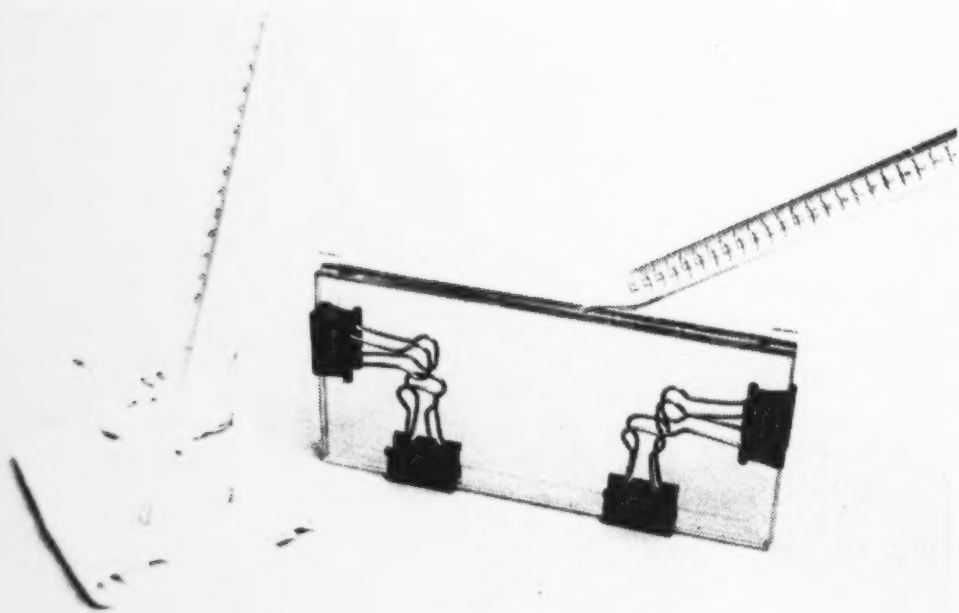


FIGURE 3. Filling the mold (see text).

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IV. PERFORMANCE OF THE AGAROSE GEL ELECTROPHORESIS TEST

- A. Fill out data sheets, including the specimen location in slits 1-13.
- B. Determine the number of agarose plates required and remove gel molds containing the agarose plates from storage.
- C. Remove agarose plates from the gel molds as follows:
 - 1. If not already done, remove clamps from the molds, and place molds on flat surface with *agarose side up*.
 - 2. Remove upper glass plate from mold by gently breaking the seal between the glass plate and the plastic U-frame; this may be accomplished by inserting a small knife blade or the edge of a small spatula between the top glass plate and the U-frame (figure 4).
 - 3. After removing the upper glass plate, gently remove the plastic U-frame without disturbing the thin layer of agarose (figure 5).
 - 4. Remove agarose plate by gently pulling it off the bottom glass plate.
- D. Place agarose plate, agarose side up, on a clean cloth or paper towel, and label (plate number, run number, date, etc.) on left edge with a marking pen.
- E. Apply a cellulose polyacetate strip on the agarose plate about one-third up from the bottom of the plate where the samples will be applied. Remove the strip immediately after it has touched the surface and has absorbed excess moisture from the gel (figure 6).

NOTE: It is important that the cellulose polyacetate strip lie perfectly flat on the agarose surface; creases may subsequently cause improper or erratic diffusion of sample proteins.

- F. Place the sample-application foil on the top of the agarose, on the same area blotted by the polyacetate strip, so that the slits are positioned in the middle of the area (figure 7). Apply a small amount of pressure to the sample-application foil to ensure that no air bubbles form beneath the foil and that it lies perfectly flat and tight on the agarose surface.



FIGURE 4. Dismantling the mold; initial step(s).

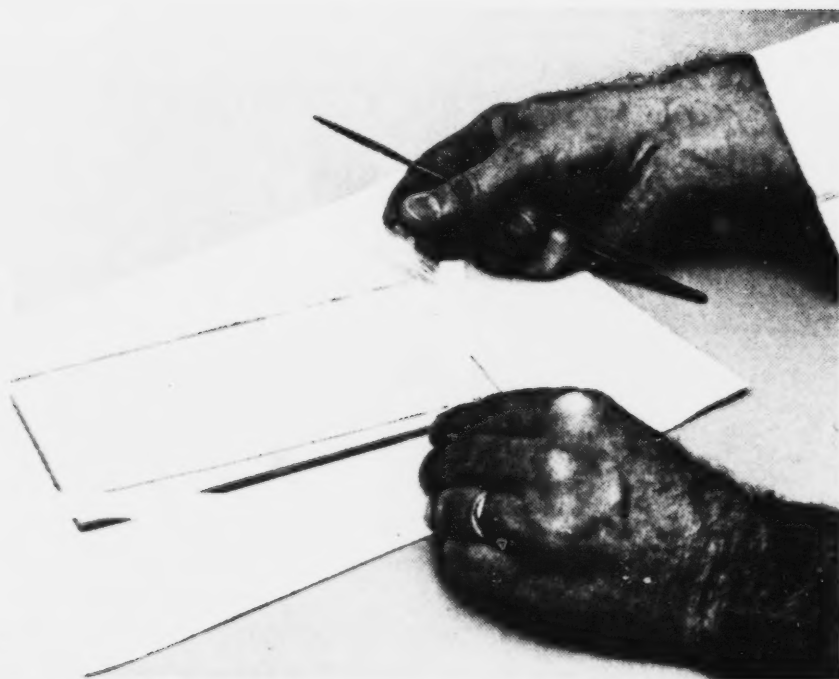


FIGURE 5. Dismantling the mold; removal of formed agarose gel plate.

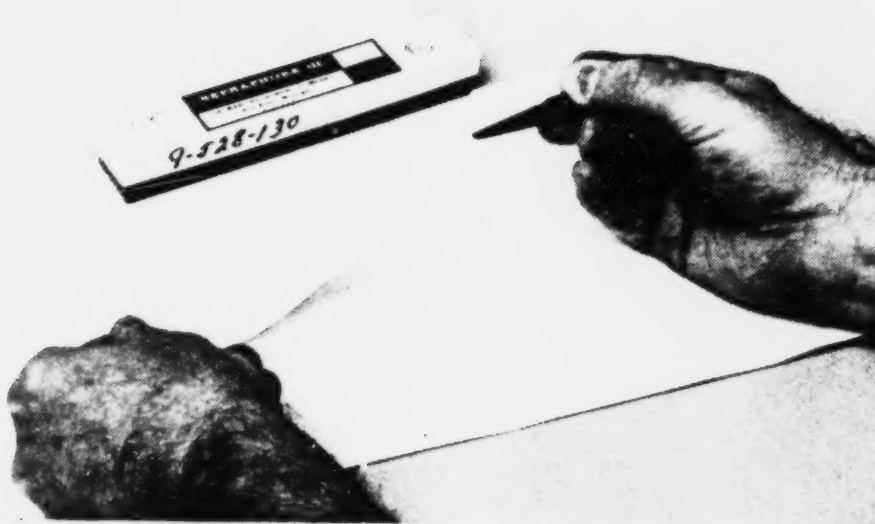


FIGURE 6. Drying of the agarose gel surface by use of a cellulose polyacetate strip.



FIGURE 7. Applying sample application foil onto the agarose gel surface.

G. Prepare electrophoresis apparatus.

1. Fill electrophoresis chamber with proper volume of half-strength barbital-HCl buffer, pH 8.6 (see manufacturer's instructions for your particular chamber), and check that the levels of buffer in both sides of the chamber are equal. The buffer can be reused several times ($\cong 10$), however the polarity of the current must be reversed after every run.
2. Connect cooling plate in the electrophoresis chamber to the thermostatic circulator and adjust temperature of circulating water to 10°-14°C (figure 8).
3. Moisten the surface of the cooling plate with distilled water.
4. Transfer the agarose plate to the cooling platform and place one edge of the agarose plate in contact with the water; then slowly lower the plate until it rests completely on the cooled surface. When the agarose plate is properly positioned on the cooling plate, a thin film of water, free of air bubbles, will remain between the polyester sheet and the cooling plate.

NOTE: The agarose plate should be positioned so that the sample-application foil is lined up with the slits on the cathode side of the electrophoresis chamber (figure 9).

- H. Align serum specimens, including normal human serum control, in the order to be added to the slits; in this case, the slits would be numbered 1 through 13, from left to right. Carefully check serum identification numbers with the data sheet.
- I. Apply 2-4 μ l of 0.4% bromphenol blue stain to some or all of the slits of the sample-application foil (Figure 10). Any excess stain deposited around the slits is absorbed by gently sweeping around the slits with blotting paper.
- J. Quickly add 2-4 μ l of each serum sample, at 5- to 10-second intervals, to its respective slit, without touching the gel surface.
- K. After a 3-minute interval, absorb any excess serum from the slits with blotting paper and carefully peel off the sample-application foil. The foil should be washed between runs in an ordinary detergent and rinsed well in isotonic saline.
- L. Quickly apply the wicks (prewet with buffer) that connect the sides of the agarose plate to the buffer vessels in both sides of

the electrophoresis chamber (figure 11). Wicks should be cut so as to cover the entire length of the agarose plate and should extend at least 10 mm onto the gel. Apply a small amount of pressure to the area of the wicks coming in contact with the gel to ensure that they are lying flat and tight on the agarose surface.

- M. If available, place anticondensation lid over the agarose plate and then position the chamber's cover.
- N. Turn on power supply to the electrophoresis chamber and adjust voltage to 15-20 v/cm, which corresponds to a total of 250-300 V and a current of 100-150 mA, depending on the type of wicks used. Allow electrophoresis to proceed for 20 minutes, or until the blue-stained albumin band has migrated approximately 3.5- to 4.0-cm from the application slit towards the anode.
- O. At the end of the electrophoresis period, turn off the power supply and thermostatic circulator, and remove agarose plate from the chamber. The agarose plate is now ready to be stained.

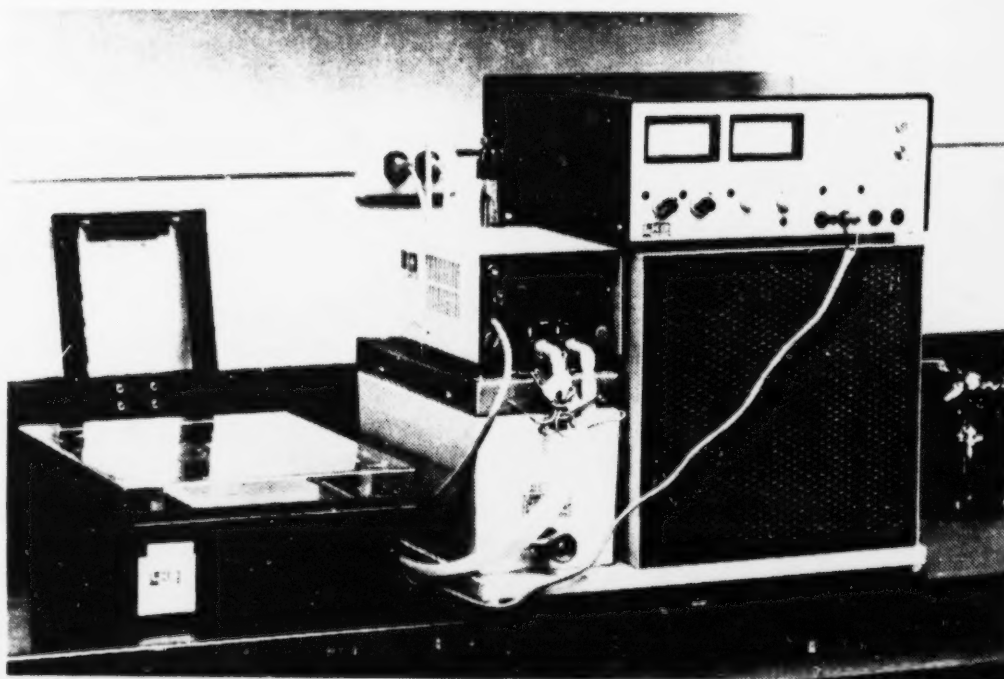


FIGURE 8. A view of the electrophoresis equipment. From left to right: the electrophoresis chamber with glass cooling plate and cover; thermostatic circulator; and direct current power supply.

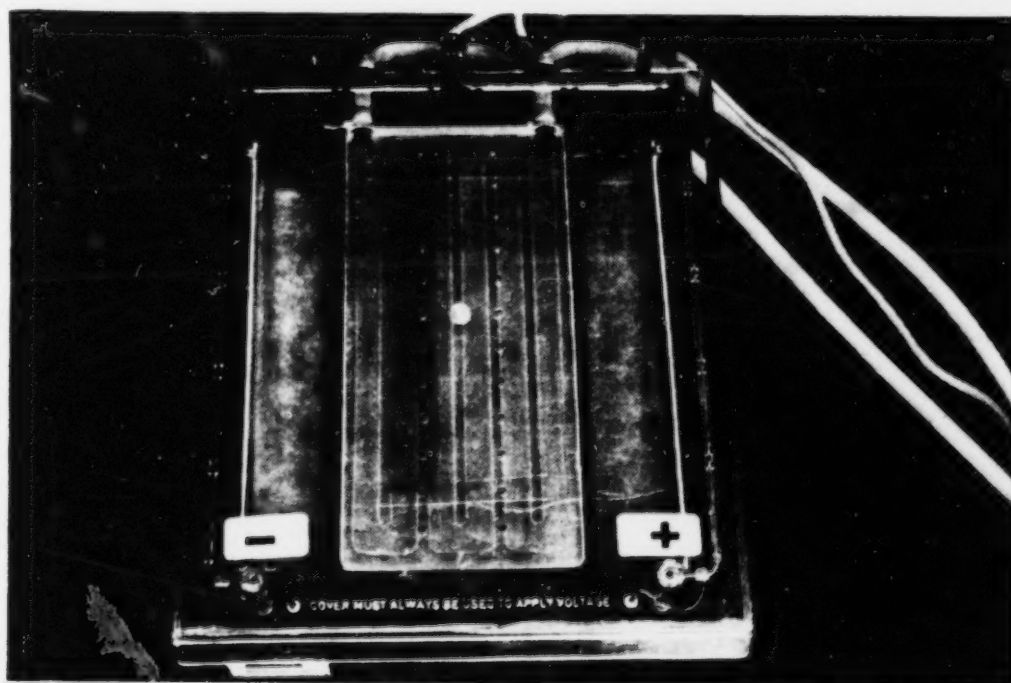


FIGURE 9. Agarose gel plate positioned on glass cooling plate of electrophoresis chamber; application foil is positioned with slits on the cathode (-) side of chamber.

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FIGURE 10. Application of 0.4% bromphenol blue stain to slits of application foil.

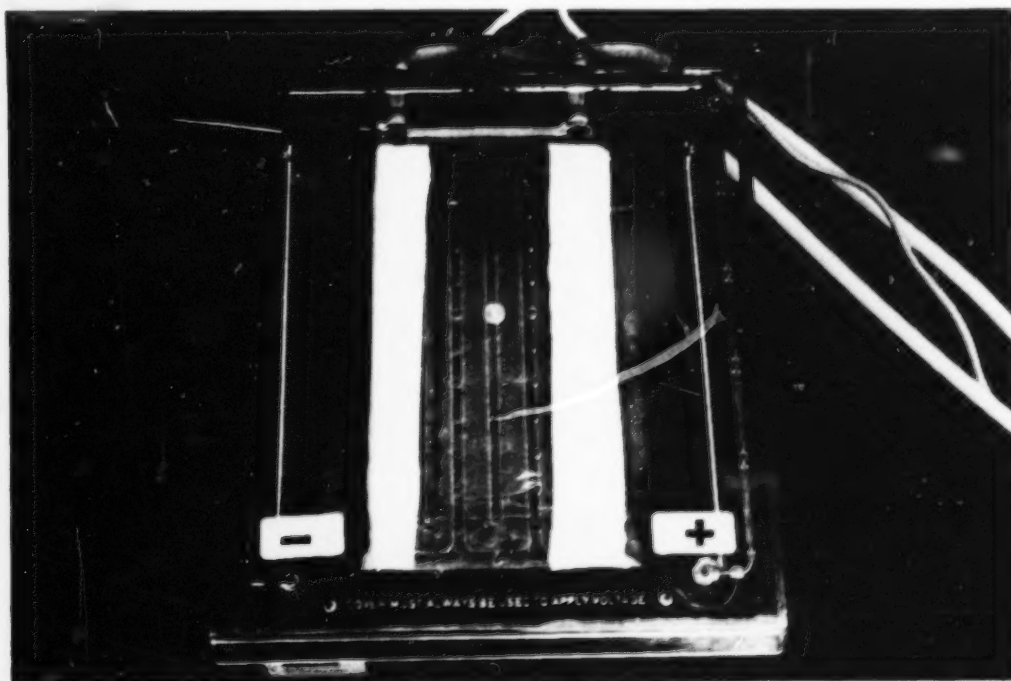


FIGURE 11. Positioning of wicks that connect agarose gel plate to buffer in both sides of electrophoresis chamber.

V. STAINING, WASHING, AND DRYING AGAROSE PLATES

- A. Fill an appropriately sized staining dish with enough Coumassie brilliant blue stain solution to completely cover the agarose plate.
- B. Immediately after termination of electrophoresis, transfer the agarose plate to the Coumassie brilliant blue stain solution. The staining solution should completely cover the plate.

NOTE: Since buffer salts will diffuse out from the agarose gel, the staining solution should be replaced after it has been used to stain six agarose plates.

- C. Stain for 30 minutes.
- D. Remove the agarose plate from the staining solution and rinse the gel in tap water.
- E. Place gel plate in a hot air oven, or direct a stream of air from a warm air blower onto the plate for quicker drying.
- F. Fill two staining dishes with enough destaining solution to completely cover the agarose plates.
- G. Place the dried agarose plate in one of the staining dishes containing destaining solution until most of the stain has been cleared off the plate.
- H. Transfer the agarose plate to the second staining dish containing destaining solution; destain until all residual background staining is cleared.
- I. Remove plate from the second destaining solution, blot gently with a paper towel and place in a hot air oven until dry.
- J. Label plate according to serum specimen slit assignment recorded on data sheet. Plates are now ready to be read.

VI. INTERPRETATION OF RESULTS

A. Introduction

The clinical use and interpretation of agarose gel electrophoresis in serum (plasma) protein analysis is based on simple electrophoretic separation of serum proteins according to their relative mobility (isoelectric points) into five major fractions: albumin, alpha-1, alpha-2, and beta- and gamma-globulins. Each of the five major fractions is composed of numerous specific protein entities. It is known that each of the classical electrophoretic zones contains two or more components in concentrations above 0.5 g per liter; however, because of their similarity in isoelectric points, multiple proteins appear as one fraction.

The agarose gel electrophoretic separation of a normal human serum is depicted in figure 12. Six stained bands differing in spread and intensity dominate the pattern. Taken in order from the anode to the cathode, these are as follows:

1. A broad, intensely stained zone of serum albumin.
2. A narrow, α -band, caused by α_1 -antitrypsin.
3. A broad zone, varying in appearance in different sera, caused mainly by α_2 -macroglobulins and haptoglobins.
4. A narrow, well-demarcated β_1 -band which is produced mainly by transferrin, and may be followed by an irregular sharp zone of β -lipoproteins.
5. A narrow β_2 -band follows. The intensity of this band reflects the concentration of the complement components.
6. A rather diffuse zone extending on both sides of the application slit which is produced by immunoglobulins. These are normally too heterogeneous to produce any distinct band but may contribute to background stain over the entire zone from α_2 to the γ -region.

Evaluation of the agarose gel electrophoresis plates is dependent upon visual inspection of the stained patterns. Under a given set of electrophoretic conditions, the intensity and position of *normal* serum protein bands are relatively consistent. The detection of abnormal bands is facilitated by comparing them with bands developed by a normal control serum tested on the same plate (see figure 12). By comparison with the normal control, the abnormalities may

appear (1) as an increase in staining intensity, accompanied by distortion of the band in the form of thickening or bulging, (2) as a decrease in staining intensity, and (3) as displacement of the band from its normal electrophoretic position; finally, there may be a complete absence of a particular band. Invariably, quantitative protein values for the major serum proteins are found to correlate with the abnormal electrophoretic patterns.

B. Patterns of Monoclonal Gammopathies

Myeloma, macroglobulinemia, and other conditions with malignant monoclonal immunocyte proliferation result in the secretion of complete, incomplete, or polymerized immunoglobulins which appear as electrophoretically discrete bands (M-components), usual-

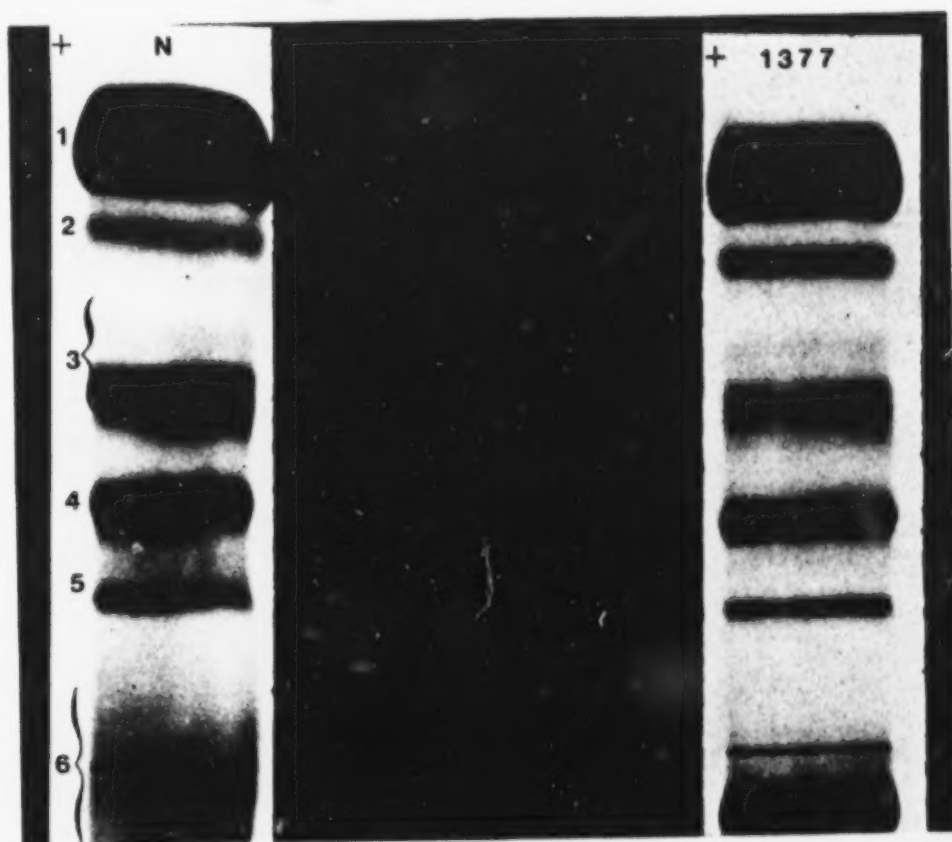


FIGURE 12. Agarose gel electrophoretic pattern of a normal human serum (N) and a serum containing an IgM monoclonal protein (1377). The appearance of the electrophoretic pattern may vary in healthy individuals due to inherited variants and to age-dependent variation in the concentration of the individual proteins.

ly in the gamma zone. However, in certain gammopathies, such as IgA myeloma or light chain disease, the abnormal bands can be found in other electrophoretic zones.

Generally, it is possible to determine if an abnormal-appearing band in the gamma zone represents a polyclonal or monoclonal cell increase because bands or partitions due to polyclonal origin always cause a more diffuse, indistinct demarcation than those resulting from monoclonal cell increase which appear as distinct, darkly stained bands. For the band(s) in question to be accepted as being of monoclonal origin, they must be identified and classified as belonging to *one* immunoglobulin class and type. This can be accomplished by immunofixation with monospecific antisera.

Representative myeloma and macroglobulinemia agarose gel electrophoresis patterns are shown in figures 13 and 14. Quantitative immunoglobulin values for the sera, as determined by radial immunodiffusion (Helena Quipate System), are given in table 1. When agarose gel electrophoresis is used as a tool for the screening of monoclonal gammopathies, certain generalizations can be made which will aid in evaluating results; these are as follows:

IgG

1. Increasing or decreasing IgG concentration may be found in the middle or cathodal gamma zone.
2. An IgG increase may involve all subclasses and thus appear as a diffuse band or may be confined to faster or slower immunoglobulin zones (fig. 14, H and I).
3. A large increase of fast, polyclonal IgG can produce a pattern that may be mistaken for an increased IgA.
4. Polyclonal immunoglobulin cell stimulation, apparent as increased staining of part or all of the gamma zone, regularly indicates increased IgG synthesis (fig. 13, 1337).

IgA

1. IgA paraprotein is seen frequently in the β_2 or α_2 - β region (fig. 14, B).
2. IgA deficiency may be suspected if the staining of the β_2 -globulin zone is weak (fig. 13, 1377), whereas a polyclonal IgA increase has the opposite effect.

TABLE 1
Quantitative Serum Immunoglobulin Values* for Figures 13 and 14

Serum	IgG	IgA	IgM
Normal Adult Range** (mg/dl)	600-1450	~ 40-350	30-220
Figure 13			
1157	1390	260	4150
532	1490	148	8860
1137	530	30	4800
1337	1950	574	35
986	880	168	306
350	949	105	595
Figure 14			
A	1070	190	84
B	550	1100	27
C	570	114	94
D	1750	160	73
E	1260	84	150
F	510	70	67
G	4130	28	41
H	1060	60	35
I	1030	15	34

*Helena Quiplate System

**See reference 14

3. IgA bands are usually much broader than IgG and IgM bands because of the presence of polymerized forms.
4. IgA bands usually possess bulging ends and appear tapered toward the anodal end.

IgM

1. M-components of the IgM class may appear on the cathodal side of the normal gamma zone.
2. High molecular weight polymers may be retained as a thin band in or on the cathodal margin of the application slit (fig. 13, 350; fig. 14, F).
3. IgM paraprotein bands are usually narrower than IgG bands and appear to have flattened ends.
4. Slow IgM components may be found from β_1 - to the slowest gamma zone.
5. M-components of the IgM class may be temperature or pH sensitive, and may be precipitated at the point of application.

Bence Jones Proteins

Bence Jones protein or light chain paraprotein can migrate more anodal than other paraproteins. The λ type may be found from the α_1 to the gamma zone, and the κ type from the α_2 to the gamma zone; they usually produce narrow bands.

C. Some General Precautions in Interpretation

1. A heavy application of serum or allowing the serum to partially dry before commencing electrophoresis will result in a thick band at the application site which may be misinterpreted as a monoclonal band.
2. Sera from patients receiving heparin therapy and sera separated from red cells before clotting is completed will contain fibrinogen. In serum electrophoresis, fibrinogen mimics a monoclonal band.
3. Serum contaminated with sialidase-producing bacteria will show a normal albumin pattern but decreased mobility of all globulin fractions in addition to IgG.
4. A common serum storage effect is the disappearance of the complement component C3 band.
5. During electrophoresis, overcooling can cause fluid drops on the gel surface which in turn can result in blurred band patterns.

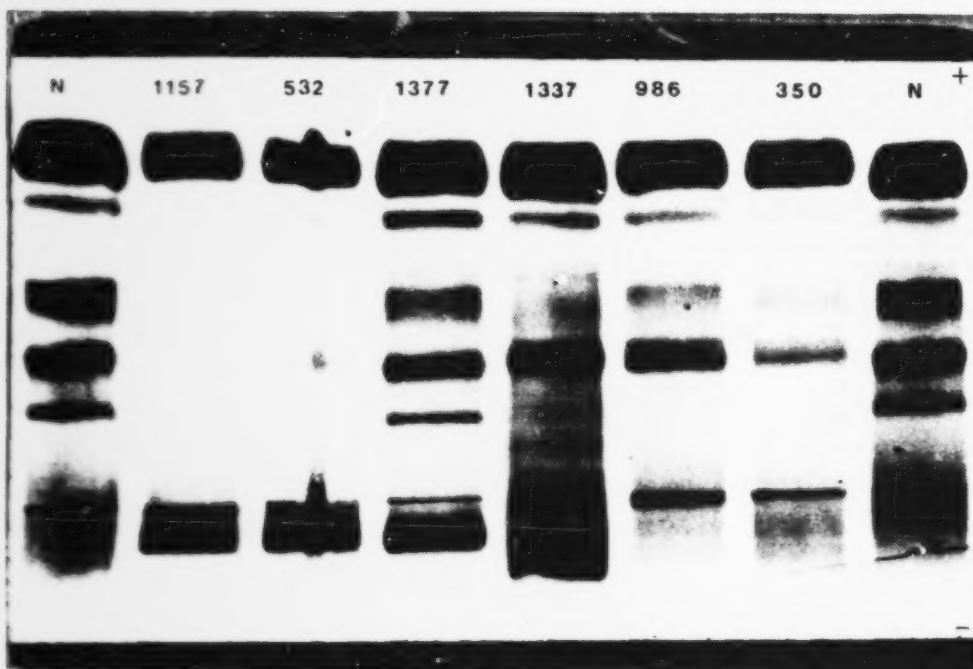


FIGURE 13. Screening electrophoresis in agarose gel of normal and pathological serum proteins.

- N — Normal pattern
- 1157 — IgM macroglobulinemia showing a monoclonal band in cathodal gamma zone; β_2 -absent
- 532 — M-component of IgM type on cathodal side of application slit; β_2 -absent
- 1377 — IgM macroglobulinemia
- 1337 — Increase of slow IgG fractions, on cathodal side of application slit, in polyclonal hypergammaglobulinemia; increase in IgA in β_2 -region
- 986 — M-component of IgM type on cathodal margin of application slit
- 350 — M-component of IgM type

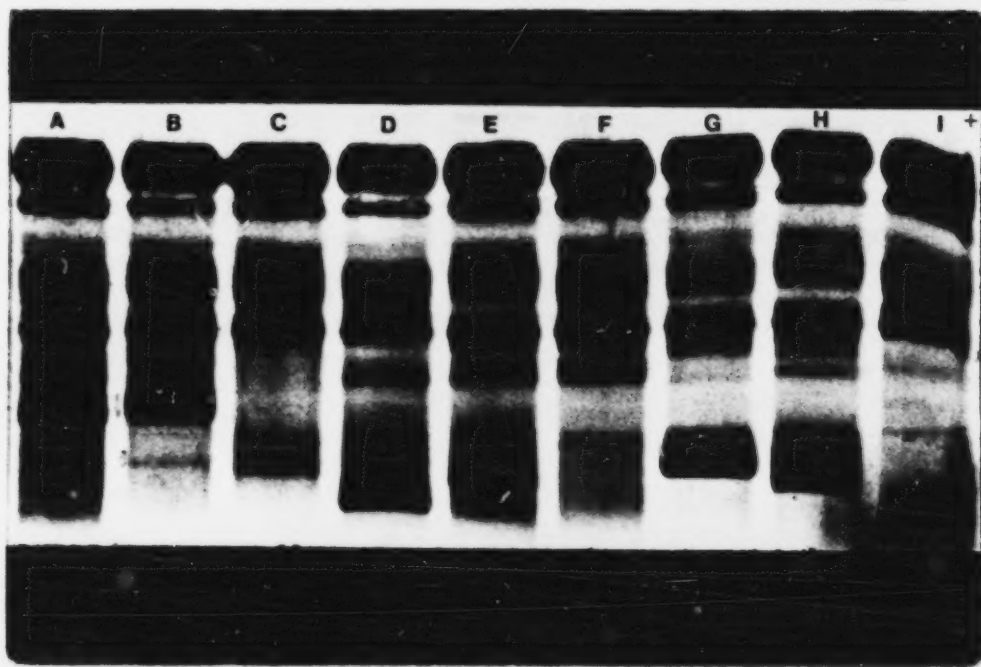


FIGURE 14. Screening electrophoresis in agarose gel of pathological serum proteins.

- A**—A modest M-component was demonstrated on serum protein electrophoresis
- B**—M-component of IgA type, on anodal side of application slit, covering the β_2 -zone; hypogammaglobulinemia involving IgG
- C**—M-component of IgG type, cathodal to application slit
- D**—Slow moving M-component of IgG type in cathodal gamma zone
- E**—M-component of IgG type, on cathodal side of application slit
- F**—Faint M-component of IgG type, on cathodal side of application slit
- G**—Strongly increased cathodal IgG M-component
- H**—Strongly increased slow moving IgG fractions, cathodal to application slit
- I**—M-component of IgG type, slow moving in cathodal gamma zone

PART B
Immunofixation Electrophoresis

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I. MATERIALS REQUIRED

A. Reagents

1. Barbitol-HCl buffer, pH 8.6
2. Agarose, electroendosmosis ($\sim$ mr) 0.13-0.19
3. Bromphenol blue stain
4. Amido black
5. Nigrosin
6. Acetic acid destain solution
7. Sera:
 - a. Normal human sera
 - b. Patients' sera
8. Antisera:
 - a. Anti-human IgG, heavy chain specific, nephelometric grade
 - b. Anti-human IgA, heavy chain specific, nephelometric grade
 - c. Anti-human IgM, heavy chain specific, nephelometric grade
 - d. Anti-human Fab (κ and λ light chains)
 - e. Anti-human lambda light chain (bound and free)
 - f. Anti-human kappa light chain (bound and free)
 - g. Anti-human free lambda light chain
 - h. Anti-human free kappa light chain
9. Normal saline, 0.85%

B. Equipment

1. 101- x 203- x 5.0-mm glass plates
2. 205- x 110- x 1.0-mm plastic U-frame (Bio-Rad Laboratories, Richmond, California)*
3. Flexible polyester film, 203-mm width, 0.1-mm-thick; one side is hydrophilic and the other is hydrophobic (GelBondTM, FMC Corporation, Marine Colloids Division, Rockland, Maine)*
4. Paper clamps for holding glass plates, polyester film, and U-frame together
5. Sample application foil (LKB Instruments, Inc., Rockville, Maryland)

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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6. Cellulose polyacetate strips, 2.5- x 15.2-cm (Gelman Sephadex III Cellulose, Gelman Sciences, Inc., Ann Arbor, Michigan)
7. Wicks, 200- x 250-mm (Ultra Wicks, Bio-Rad Laboratories, Richmond, California)
8. Blotting paper
9. Capillary tubes
10. Erlenmeyer flasks, screw cap, 500-ml, 1000-ml, 2000-ml
11. Serologic pipettes, 5-ml, 10-ml, 25-ml
12. Graduated cylinders, 100-ml, 500-ml
13. Volumetric flasks, glass stopper, 500-ml, 1000-ml, 2000-ml
14. Covered staining trays
15. Magnetic stirrer and bars
16. Tripod with asbestos pad
17. Bunsen burner

NOTE: Alternately, an electric heater with magnetic stirring may be used in place of items 15-17.

18. Interval timer
19. Hot air drying oven
20. Marking pen
21. Test-tube racks
22. Electrophoresis apparatus
 - a. Electrophoresis chamber, with a glass cooling plate.
 - b. Thermostatic circulator with circulating flow rate of 1-2 liters/min. and adjustable between 0°-20°C.

NOTE: Alternately, cooling of the glass plate with circulating water from a cold water tap can be used.

- c. Power supply, with a capacity of 300 V and capacity of 150 mA per electrophoresis cell.
23. Refrigerator, 4°C

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II. PREPARATION OF REAGENTS

A. Barbital-HCl Buffer, pH 8.6

Chemicals needed:

Sodium barbital ($\text{NaC}_8\text{H}_{11}\text{N}_2\text{O}_3$)

Calcium lactate $\text{Ca}(\text{C}_3\text{H}_5\text{O}_3)_2 \cdot 5\text{H}_2\text{O}$

1N HCl

Distilled water

1. Prepare stock solutions as follows:

Solution A:

- a. Add approximately 1000 ml of distilled water to a 2-liter volumetric flask and heat over a Bunsen burner or electric heater until water is near boiling.
- b. Add 206.18 gm of sodium barbital to the flask and mix thoroughly to dissolve.
- c. Add distilled water (q.s.) to 2 liters and allow solution to cool to room temperature. This solution may be stored in a tightly closed bottle at room temperature ($20^\circ\text{-}26^\circ\text{C}$) for several weeks; however, storage at 4°C is preferable. Discard at the first sign of contamination.

Solution B:

- a. Add 86.2 ml of 11.6N HCl to a 1-liter volumetric flask.
- b. Add distilled water (q.s.) to 1 liter, stopper, and mix thoroughly. After mixing, a solution of 1N HCl is obtained.

NOTE: A HCl solution, at a concentration of 1N, may be obtained from a commercial source.

2. Prepare half-strength barbital-HCl buffer, pH 8.6, as follows:

- a. Add 0.62 gm of calcium lactate to a 2-liter volumetric flask.
- b. Add 174.2 ml of solution A and 12.9 ml of solution B to the flask. Mix thoroughly to dissolve the calcium lactate.
- c. Add distilled water (q.s.) to 2 liters, stopper, and mix.
- d. Check the pH with a prestandardized pH meter. The pH should be 8.6 ± 0.05 . If the pH is below 8.55, adjust with 1N NaOH. If the pH is above 8.65, adjust with 1N HCl.

- e. Store this final *working buffer* in a tightly closed bottle at 4°C. It may be stored for several weeks, but check pH before using. Discard at the first sign of contamination or change in pH.

B. 1.2% Agarose in Barbital-HCl Buffer

Chemicals needed:

Agarose, electroendosmosis (-mr) 0.13-0.19

Barbital-HCl buffer, pH 8.6

1. Add 98.8 ml *half-strength barbital-HCl buffer* to a 250-ml Erlenmeyer flask.
2. Add 1.2 gm of agarose to the buffer.
3. Place the flask on an asbestos pad over a Bunsen burner, or on an electric heater with magnetic stirring, and heat the solution until agarose is completely dissolved, as indicated by complete clarity of the solution.

NOTE: Too much heating will turn the solution yellow. Keep the flask loosely covered during heating to retard evaporation. Once agarose is in solution, remove flask from heat and let cool to approximately 60°C before pouring plates. If the solution is too hot it will cause the polyester film to buckle and warp.

C. Amido Black/Nigrosin Stain

Mix:

Amido Black	0.5 gm
Nigrosin	0.5 gm
Glacial acetic acid	50.0 ml
Distilled water	950.0 ml

Filter the solution and store at room temperature in a tightly closed bottle.

D. 0.4% Bromphenol Blue Stain Solution

Mix:

Bromphenol blue	0.4 gm
Barbital-HCl buffer, pH 8.6, half-strength	100.0 ml

E. Destaining Solution, 5% Acetic Acid

Mix:

Glacial acetic acid	50.0 ml
Distilled water	950.0 ml

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III. PREPARATION OF AGAROSE PLATES

- A. Determine the number of plates needed to perform the immunofixation test as follows:
1. Count the number of patients' sera to be tested.
 2. Three patients' sera, or two patients' sera and a normal human serum control, can be screened on one plate. Therefore, divide the *total* number of sera to be tested by three to obtain the number of plates needed.
- B. Estimate the volume of 1.2% agarose solution needed for the number of plates determined above, as follows:
1. Each plate is layered with 15 ml of 1.2% agarose. Therefore, multiply the number of plates to be made by 15 to determine the volume of agarose needed.
 2. Increase the volume obtained in the step above by a few milliliters to facilitate pipetting.
- C. Prepare the desired volume of 1.2% agarose in barbital-HCl buffer, as described on page 6.
- D. For each plate to be poured, prepare a gel mold as follows (fig. 1).
1. Cut a piece of GelBond™ polyester film, 115- x 203-mm for each plate.
 2. Set aside 2, 101- x 203- x 5.0-mm glass plates for each agarose plate to be prepared.
 3. Wet one side of a glass plate with a small volume of distilled water and place a precut sheet of GelBond™ polyester film on the wet plate with its *hydrophilic side up*.
- NOTE:** This is tested by noting the behavior of a drop of water on both sides of the film; the water will "bead" on the hydrophobic side, whereas it will "spread" on the hydrophilic side.
- No air bubbles should remain between the glass plate and the polyester sheet.
4. Place a plastic U-frame on top of the sheet and then the second glass plate, and clamp them together with strong paper clamps, along the bottom and the edges so that you have a mold, from bottom to top, consisting of a glass plate, polyester film, a plastic U-frame, and a second glass plate.

- E. Position the gel mold vertically on a flat surface with the open end of the mold up (figure 2).
- F. Light a Bunsen burner and place it close to the working area.
- G. Prewarm a 25-ml pipette by passing it through the flame of the burner and draw up 15 ml of the hot 1.2% agarose which has been allowed to cool to 60°C.
- H. Quickly pipette the agarose into the mold. Keep the pipette at a 45°C angle to the level of the mold and allow the agarose to flow out in one step (5-10 seconds) as a continuous stream between the GelBond™ film and the top glass plate. Any air bubbles should move rapidly upwards as the mold is filled (figure 3).
- I. Allow the agarose to solidify, approximately 20 minutes, at room temperature.
- J. Trim excess GelBond™ film from upper end of the gel mold and seal the open upper end of the mold with waterproof tape. The two glass plates and plastic U-frame must remain intact until the agarose plate (GelBond™ sheet layered with 1.2% agarose, 1.0-mm thick) is ready for use.
- K. Place intact gel molds in a water-saturated atmosphere (humidity chamber) and store at 4°C for at least 16 hours before use. Agarose plates can be stored for at least 1 week in a humidity chamber at 4°C.

NOTE: An alternate storage procedure: Remove the clamps from the gel molds, individually wrap them tightly with a wet paper towel and plastic wrap, and store them at 4°C.

IV. PERFORMANCE OF THE IMMUNOFIXATION TEST

A. Sample preparation

Serum, plasma, urine, other body fluids, or protein fractions may be assayed by immunofixation. Immunofixation is best performed with modest-to-marked antibody excess. Excess antigen, for example, can result in fixed bands with unstained centers (see fig. 16). Therefore, when applying immunofixation to the study of human monoclonal proteins, the serum and urine samples must be diluted with buffer (barbital-HCl) as follows:

1. *Suspected IgG, IgA, or IgM monoclonal protein*; the serum is diluted so as to have the concentration of the monoclonal protein adjusted to 200.0 mg/dl.

NOTE: The concentration of suspected monoclonal proteins, as noted following standard serum protein electrophoresis or agarose gel electrophoresis, in a patient's serum (IgG, IgA, IgM), may be determined by (1) radial immunodiffusion, (2) rocket immunoelectrophoresis, or (3) nephelometry.

Example: The concentration of IgG in a patient's serum is determined to be 4,000.0 mg/dl. Therefore, for immunofixation this serum would be diluted 1:20 with half-strength barbital-HCl buffer to adjust the concentration of IgG to approximately 200.0 mg/dl.

2. *Suspected IgD or IgE monoclonal protein*; generally, the serum is tested undiluted. However, if quantitative data indicates either of these proteins to be present at a concentration greater than 200 mg/dl the serum will need to be diluted to adjust the concentration to 200.0 mg/dl.
3. *Urine samples.* The same urine sample as prepared for agarose gel electrophoresis, a 100X concentrated sample, is used in immunofixation. This urine sample is tested either undiluted (i.e., as a 100X concentrate) or diluted further with buffer, depending upon the intensity of the urinary light chain bands produced after electrophoresis. The dilution scheme for a urine sample, starting with the 100X concentrated sample, is as follows:
 - a. A 1:5 dilution, if a medium band was seen following electrophoresis (final dilution, 1:500).

- b. A 1:10 dilution, if a heavy band was produced by electrophoresis (final dilution, 1:1000).
 - c. Undiluted (100X concentrate), if electrophoresis produced only a faint band.
- B. Fill out data sheets, including the specimen location in slits 1-13.
- C. Determine the number of agarose plates required and remove gel molds containing the agarose plates from storage.
- D. Remove agarose plates from the gel molds as follows:
 - 1. If not already done, remove clamps from the molds, and place molds on flat surface with *agarose side up*.
 - 2. Remove upper glass plate from mold by gently breaking the seal between the glass plate and the plastic U-frame; this may be accomplished by inserting a small knife blade or the edge of a small spatula between the top glass plate and the U-frame (figure 4).
 - 3. After removing the upper glass plate, gently remove the plastic U-frame without disturbing the thin layer of agarose (figure 5).
 - 4. Remove agarose plate by gently pulling it off the bottom glass plate.
- E. Place agarose plate, agarose side up, on a clean cloth or paper towel, and label (plate number, run number, date, etc.) on left edge with a marking pen.
- F. Apply a cellulose polyacetate strip on the agarose plate about one-third up from the bottom of the plate where the samples will be applied. Remove the strip immediately after it has touched the surface and has absorbed excess moisture from the gel (figure 6).

NOTE: It is important that the cellulose polyacetate strip lie perfectly flat on the agarose surface; creases may subsequently cause improper or erratic diffusion of sample proteins.
- G. Place the sample-application foil, containing thirteen application slits, on the top of the agarose, on the same area blotted by the polyacetate strip, so that the slits are positioned in the middle of the area (figure 7). Apply a small amount of pressure to the sample-application foil to ensure that no air bubbles form beneath the foil and that it lies perfectly flat and tight on the agarose surface.

H. Prepare electrophoresis apparatus

1. Fill electrophoresis chamber with proper volume of half-strength barbital-HCl buffer, pH 8.6 (see manufacturer's instructions for your particular chamber), and check that the levels of buffer in both sides of the chamber are equal. The buffer can be used several times (≈ 10); however, the polarity of the current must be reversed before every run.
2. Connect cooling plate in the electrophoresis chamber to the thermostatic circulator and adjust temperature of circulating water to 10°-14°C (figure 8).
3. Moisten the surface of the cooling plate with distilled water.
4. Transfer the agarose plate to the cooling platform and place one edge of the agarose plate in contact with the water; then slowly lower the plate until it rests completely on the cooled surface. When the agarose plate is properly positioned on the cooling plate, a thin film of water, free of air bubbles, will remain between the polyester sheet and the cooling plate.

NOTE: The agarose plate should be positioned so that the sample-application foil is lined up with the slits on the cathode side of the electrophoresis chamber (figure 9).

- I. Align serum specimens in the order to be added to the slits; in this case, the slits are numbered 1 through 13, from left to right. Carefully check serum identification numbers with the data sheet.
- J. Apply 2-4 μ l of 0.4% bromphenol blue stain to some or all of the slits of the sample-application foil (figure 10). Any excess stain deposited around the slits is absorbed by gently sweeping around the slits with blotting paper.
- K. Working rapidly, add 2-4 μ l of each serum sample, at 5- to 10-second intervals, to its respective slit without touching the gel surface.
- L. After a 3-minute interval, absorb any excess serum from the slits with blotting paper, and carefully peel off the sample-application foil. The foil should be washed between runs in an ordinary detergent and rinsed well in isotonic saline.
- M. Quickly apply the wicks (prewet with buffer) that connect the sides of the agarose plate to the buffer vessels in both sides of the electrophoresis chamber (figure 11). Wicks should be cut so

as to cover the entire length of the agarose plate and should extend at least 10 mm onto the gel. Apply a small amount of pressure to the area of the wicks coming in contact with the gel to ensure that they are lying flat and tight on the agarose surface.

- N. If available, place anticondensation lid over the agarose plate, and then position the chamber's cover.
- O. Turn on power supply to the electrophoresis chamber and adjust voltage to 15-20 V/cm, which corresponds to a total of 250-300 V and a current of 100-150 mA, depending on the type of wicks used. Allow electrophoresis to proceed for 20 minutes, or until the blue-stained albumin band has migrated approximately 3.5 to 4.0 cm from the application slit towards the anode.
- P. Prepare cellulose acetate strips, 1- x 4-cm, and store in covered Petri dish.
- Q. Align antiserum vials.
- R. At the end of the electrophoresis period, turn off the power supply and thermostatic circulator, and remove agarose plate from the chamber. The agarose plate is now ready for immunofixation.
- S. Immediately after electrophoresis, dip cellulose acetate strips into appropriate undiluted nephelometric-grade antiserum, drain against the side of the vial, and carefully apply strips to the gel surface so that no air bubbles are trapped beneath the strips and adjacent strips are not touching. The strips are placed so as to cover the appropriate zone known to contain the particular protein under study (figure 15). Location of the zone is determined by referring to the stained agarose-gel electrophoresis plate.

Example: The suspected monoclonal protein is IgG. Therefore, the following antisera would be used:

- a. Anti-human IgG, heavy chain specific (FC)
- b. Anti-human Fab (κ and λ light chain)
- c. Anti-human kappa light chain (bound and free)
- d. Anti-human lambda light chain (bound and free)

If the presumed IgG band was observed below the application point (slit) after the initial agarose-gel electrophoresis, the cellulose acetate strips saturated with monospecific antisera would be applied to the gel surface so as to cover the same zone.

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- T. Place agarose plates in a humidity chamber and allow to incubate at room temperature for *at least* 30 minutes. The incubation temperature is not critical.
- U. At completion, with the aid of tweezers, carefully remove and discard the cellulose acetate strips. The agarose plate is now ready to be stained.

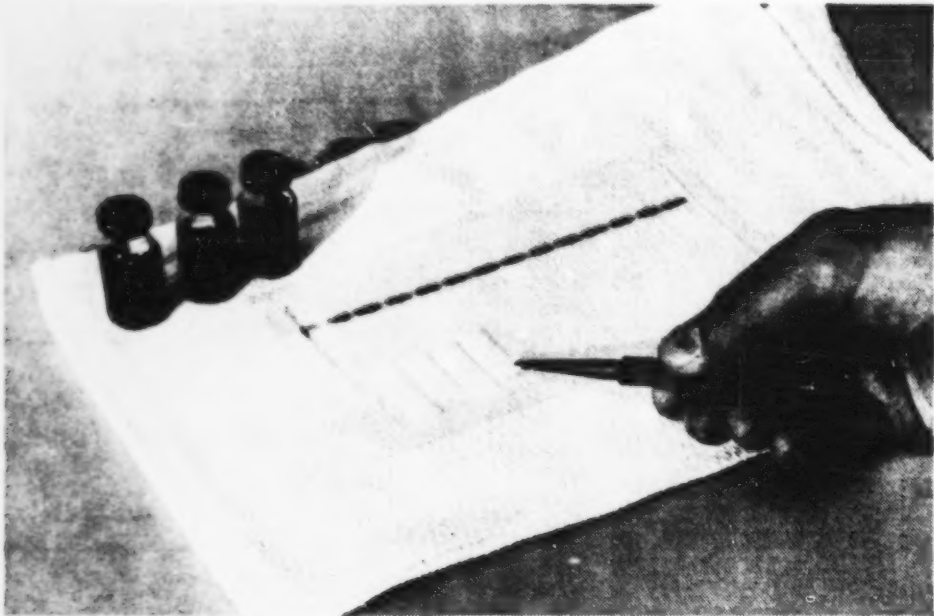


FIGURE 15. Positioning cellulose acetate strips saturated with monospecific antisera onto agarose gel surface after electrophoresis.

V. WASHING, DRYING, AND STAINING IMMUNOFIXATION PLATES

- A. After completing the incubation period and removing the cellulose acetate strips, cover the entire gel film of the immunofixation plate with a layer of filter paper which has been moistened with 0.85% saline solution.
- B. Overlay the saline-moistened filter paper with 3-5 dry absorbent paper towels (a 2- to 3-cm layer) and cover with a glass or plastic plate that will withstand and distribute a weight of 5 kg over an area of 10 x 20 cm.
- C. Position a weight of 5 kg on the glass or plastic plate and maintain this pressure for 10 minutes.
- D. At completion, remove the pressure, absorbent paper, and filter paper and transfer the immunofixation plate to an appropriately sized staining dish containing 1 liter of 0.85% saline solution. The saline should completely cover the plate.
- E. Wash plate overnight.
- F. Remove plate from the saline solution and cover the entire gel with a layer of filter paper.
- G. Place plate in a hot air oven or direct a stream of air from a warm air blower onto the plate for quicker drying.
- H. Fill an appropriately sized staining dish with enough amido black/nigrosin stain solution to completely cover the immunofixation plate.
- I. Stain for 10-20 minutes.
- J. Fill two staining dishes with enough destaining solution (5% acetic acid) to completely cover the immunofixation plates.
- K. Place the stained plate in one of the staining dishes containing destaining solution until most of the stain has been cleared off the plate.
- L. Transfer the plate to the second staining dish containing destaining solution; destain until all residual background staining is cleared.

- M. Remove plate from the second destaining solution; blot gently with a paper towel.
- N. Label plate according to serum specimen slit assignment recorded on data sheet. Plates are now ready to be read.

VI. INTERPRETATION OF RESULTS

Immunofixation electrophoresis patterns can be readily interpreted because the only immunoglobulin or immunoglobulin subunit bands stained are those which have reacted with the specific antisera used. Under most circumstances, the normal immunoglobulins present in the serum are of lower concentration than the monoclonal protein and after the serum is routinely diluted their concentration is too low for detection. Thus, the formation of an immunoprecipitin band with the specific antiserum identifies the protein of interest. In addition, because electrophoresis for immunofixation is performed exactly as for routine agarose-gel electrophoresis staining, bands made visible by both methods can be compared easily.

In determining which heavy chain specific antisera (γ , α , or μ , and in some instances, σ or ϵ) are to be used in immunofixation, use is made of the quantitative immunoglobulin data for each serum and the electrophoretic position of the abnormal band found after routine agarose-gel electrophoresis. The quantitative data are used also to determine the dilution of the particular serum needed to have the concentration of the suspected monoclonal protein adjusted to 200.0 mg/dl before electrophoresis. For each serum specimen, in addition to the heavy-chain specific antiserum, anti-Fab antiserum, and specific kappa (κ) and lambda (λ) antisera are used in the immunofixation run.

The anti-Fab antiserum serves as a useful cross-check on the reactions with the anti-kappa and anti-lambda antisera. The precipitin band resulting from immunofixation with anti-Fab antiserum should appear in the same electrophoretic position as the band produced with the anti-heavy chain monospecific antiserum. In turn, immunofixation with anti-kappa and anti-lambda antisera should result in a precipitin band (either with anti-kappa *only* or with anti-lambda *only* if typing a monoclonal protein) which parallels the electrophoretic position of the bands resulting from immunofixation with the anti-heavy chain monospecific and anti-Fab antisera.

Occasionally, after immunofixation, some myeloma proteins will give a reaction with the monospecific anti-heavy chain antiserum and anti-Fab antiserum as expected, but will show a barely discernible precipitin band or no reactivity with either of the specific anti-light chain antisera. Failure of the specific anti-light chain antisera to react appropriately could be misinterpreted as the possible existence of a heavy chain disease if the anti-Fab antiserum is not included in

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the immunofixation run. The fact that a precipitin band does result from reaction with the anti-Fab antiserum precludes such a diagnosis. Generally, in these instances, the nonreactivity of the specific anti-light chain antisera is attributable to individual antigenic variation(s) in the light chain of the myeloma protein which are not recognized by most available anti-kappa or anti-lambda antisera. (This is most commonly seen with IgA myeloma, lambda type.) On the other hand, the anti-Fab antiserum has a wider specificity than the specific anti-light chain antisera and is able to react with the light chain in question.

Three examples of immunofixation electrophoresis results, together with the agarose gel electrophoresis pattern for each serum, are presented.

The original agarose-gel electrophoresis pattern for this serum (shown on right side, fig. 16) showed a markedly increased cathodal M-component. Quantitative immunoglobulin data revealed an increased IgG level (4130 mg/dl), together with decreased IgA (28 mg/dl) and IgM (41 mg/dl) values. Such data are consistent with multiple myeloma of the IgG type. Before electrophoresis, the serum was diluted 1:20 to reduce the concentration of IgG to approximately 200 mg/dl.

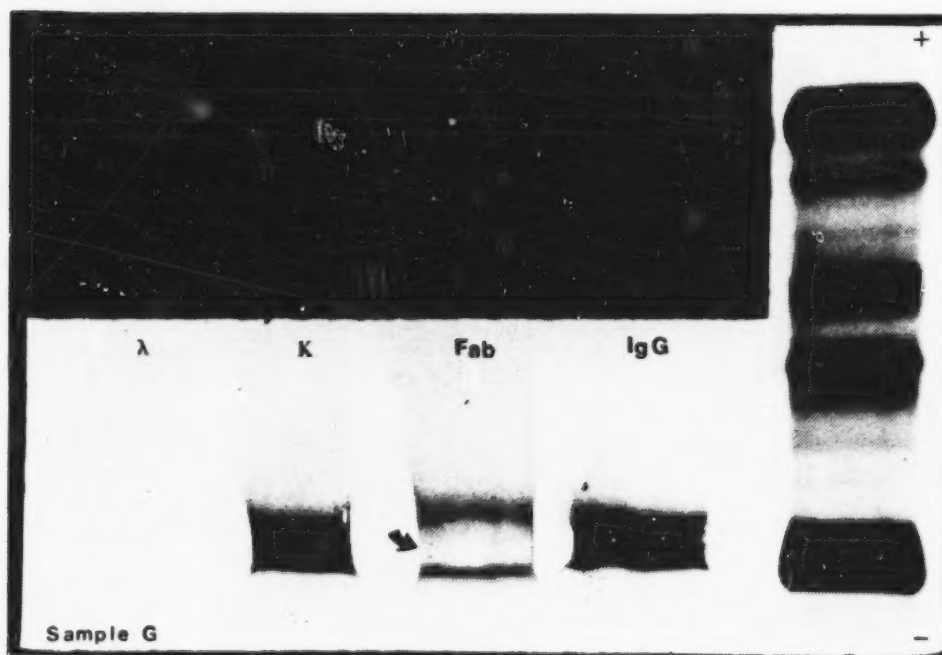


FIGURE 16. Immunofixation; IgG monoclonal protein of kappa-type light chain.

Immunofixation, with IgG heavy chain (γ) monospecific antiserum, resulted in an immunoprecipitin reaction. The precipitin band appeared in the same electrophoretic position as seen with the original agarose-gel electrophoresis pattern. Exposure of this serum to anti-Fab antiserum and both lambda (λ) and kappa (κ) light chain specific antisera resulted in a precipitin band with the anti-Fab antiserum, and with *only* the κ -antiserum; this would indicate a monoclonal type protein. Therefore, the apparent diagnosis of this serum is that it contained an IgG myeloma protein with κ -light chains, GMP(κ).

NOTE: In immunofixation, it is not uncommon to find that when a monoclonal protein is too concentrated for the antiserum applied or it is somewhat antigenically different, a precipitin band is formed which has a central clear zone of antigen excess, bordered by two well-defined zones. See figure 16 (arrow).

The original agarose-gel electrophoresis pattern for this serum (left side, fig. 17) showed an increased M-component on anodal side of application site, extending into the β_2 zone. Quantitative immunoglobulin data revealed an increased IgA level (2600 mg/dl) together with decreased IgG (<422 mg/dl) and IgM (<50 mg/dl)

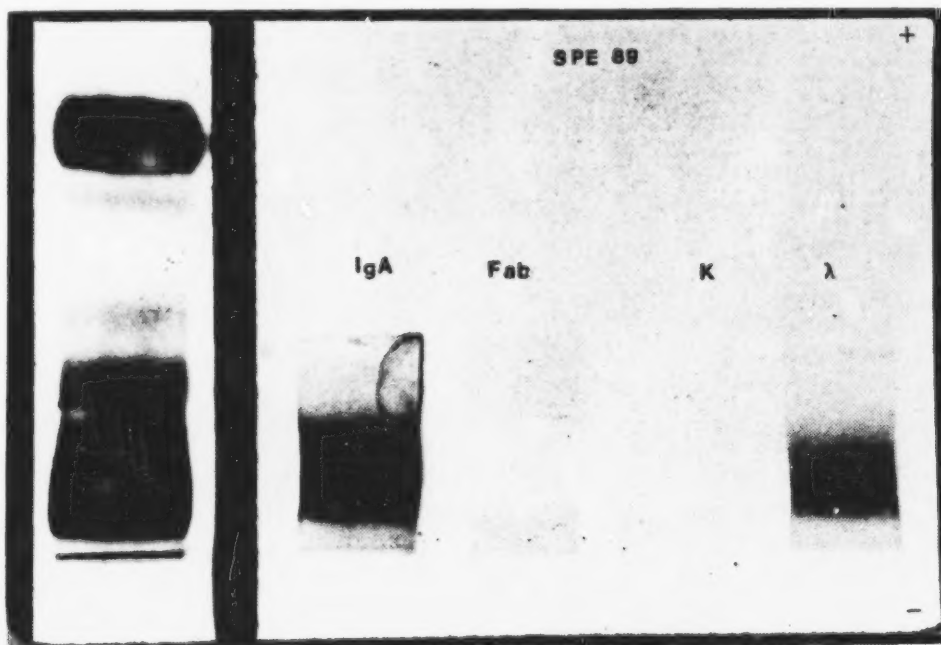


FIGURE 17. Immunofixation; IgA monoclonal protein of lambda-type light chains.

values. Such data are consistent with multiple myeloma of the IgA type. Before electrophoresis, the serum was diluted 1:13 to reduce the concentration of IgA to approximately 200 mg/dl.

Immunofixation, with IgA heavy chain (α) monospecific antiserum, resulted in an immunoprecipitin reaction. The precipitin band appeared in the same electrophoretic position as seen with the original agarose-gel electrophoresis pattern. Exposure of this serum to anti-Fab antiserum and both λ and κ light chain specific antisera resulted in a faint precipitin band with the anti-Fab antiserum (not visible in the photograph), and with *only* the λ -antisera; this would indicate a monoclonal type protein. Therefore, the apparent diagnosis of this serum is that it contained an IgA myeloma protein with λ -light chains, AMP(λ).

The original agarose-gel electrophoresis pattern for this serum (right side, fig. 18) showed an intensely stained abnormal band on cathodal side of application slit. Quantitative immunoglobulin data revealed a markedly increased IgM level (4800 mg/dl), together with decreased IgG (530 mg/dl) and IgA (30 mg/dl) values. These values are consistent with Waldenström's macroglobulinemia. Before electrophoresis, the serum was diluted 1:24 to reduce the concentration of IgM to approximately 200 mg/dl.

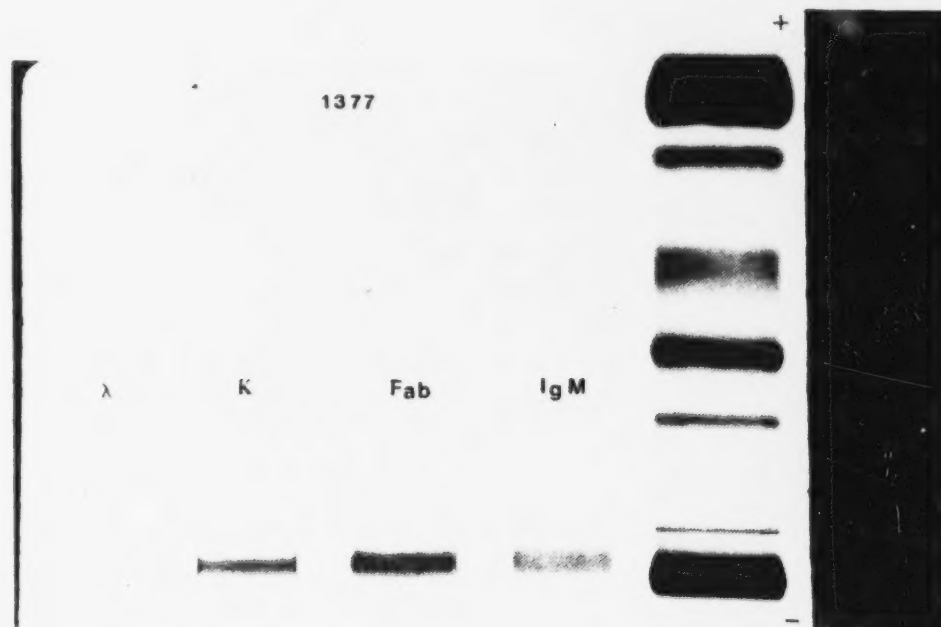


FIGURE 18. Immunofixation; IgM monoclonal protein of kappa-type light chains.

Immunofixation, with IgM heavy chain (μ) monospecific antiserum, resulted in an immunoprecipitin reaction. The precipitin band appeared in the same electrophoretic position as seen with the original agarose-gel electrophoresis pattern. Exposure of this serum to anti-Fab antiserum and both λ and κ light chain specific antisera resulted in a precipitin band with the anti-Fab antiserum, and with *only* the κ -antisera; this would indicate a monoclonal type protein. Therefore, the apparent diagnosis of this serum is that it contained an IgM monoclonal protein (Waldenström macroglobulin) with κ -light chains.

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